DISSERTATION ON LYMPHOEPITHELIAL CARCINOMA WITH SPECIAL REFERENCE TO NASOPHARYNX

Dissertation submitted to

THE TAMILNADU
DR. M.G.R. MEDICAL UNIVERSITY

in partial fulfillment for the award of the degree of

DOCTOR OF MEDICINE
IN
PATHOLOGY

STANLEY MEDICAL COLLEGE
CHENNAI – TAMIL NADU

MARCH 2010

CERTIFICATE

This is to certify that the dissertation entitled, **"DISSERTATION ON LYMPHOEPITHELIAL CARCINOMA WITH SPECIAL REFERENCE TO NASOPHARYNX"** submitted by **Dr.T.SUBACHITRA** in partial fulfillment for the award of the degree of Doctor of Medicine in Pathology by The Tamil Nadu Dr.M.G.R.Medical University, Chennai is a bonafide record of the work done by her in the Department of Pathology, Stanley Medical College, Chennai, during the academic year 2007 – 2010.

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ACKNOWLEDGEMENT

I take this opportunity to express my heart felt gratitude to **Dr.S.Chitra, M.D., Phd.,** Professor and Head of the Department of Pathology, Stanley Medical college, Chennai for her keen interest, constant encouragement , guidance and valuable suggestions throughout this study.

I take this opportunity to express my heart felt gratitude to **Dr. A.Sundaram, M.D.,** Director, Institute of Pathology, Madras Medical college, Chennai for his keen interest, constant encouragement , guidance and valuable suggestions throughout this study.

I am extremely thankful to **Dr. R. Geetha, M.D.,** Professor of Pathology, Stanley Medical college who has extended her encouragement, guidance and valuable suggestions throughout the study.

My sincere thanks to **Dr. V. Ramamoorthy, M.D.,** Professor of Pathology, Stanley Medical college for the encouragement and guidance extended to me during the study.

I am extremely thankful to **Dr. R. Padmavathy, M.D.,** Professor of Pathology, Stanley Medical college who has extended her encouragement , guidance and valuable suggestions throughout the study.

My heartfelt thanks to **Dr. P. Arunalatha, M.D.,** Professor of Pathology, Stanley Medical college for the encouragement and guidance extended to me during the study.

My sincere thanks to **Dr.K. Karkuzhali, M.D.,** Professor of Pathology, Madras

Medical college for the encouragement and guidance extended to me during the study.

My heartfelt thanks to **Dr.V. Rajalaksmi, M.D.,** Professor of Pathology, Kilpauk Medical college for the encouragement and guidance extended to me during the study.

My heartfelt thanks to **Dr. Vasanthi M.D.,** Professor of Pathology, Govt. Royapettah Hospital for the encouragement and guidance extended to me during the study.

Last but not the least I am grateful to all the faculty members, my colleagues and the technical staff members of the Department of Stanley Medical College and **my beloved family members** for their constant support and encouragement during the period of study.

CONTENTS

ABBREVIATIONS

AR	-	Antigen Retrieval
EBV	-	Epstein Barr Virus
DAB	-	Diamino benzidine
DPX	-	Distrene dibutyl pthalide in xylol
H & E	-	Hematoxylin and Eosin
HPE	-	Histopathological Examination
HRP	-	Horse radish peroxidase
IHC	-	Immunohistochemistry
LEC	-	Lymphoepithelial Carcinoma
LMP	-	Latent Membrane Protein
NPC	-	Nasopharyngeal Carcinoma
SS Label	-	Sensitive Secondary Label
WHO	-	World Health Organization

INTRODUCTION

Lymphoepithelial carcinoma (LEC) is a unique subset of head and neck squamous cell carcinoma, with a marked geographical variation is shown in incidence. Although LEC is rare worldwide,it is one of the most common cancers in South East Asian countries as well as in China,where it has an incidence of 20-50 per 100 000 individuals[1].

The incidence of LEC in the Indian subcontinent is not well documented ,but it seems to show geographical variation.LEC is generally rare in India as compared to South East Asian countries,with the exception of some northeastern states, mainly Nagaland[2].The present study compares the incidence ,age,sex distribution and relationship of EBV and LEC in sporadic cases of LEC from India with world statistics.

Lymphoepithelial carcinoma is an undifferentiated neoplasm arising in the nasopharyngeal mucosa. It is remarkable for the striking geographic difference in incidence as well as near consistent association with Epstein – Barr virus. LEC shows bimodal distribution with peaks in younger and older age groups. It shows male predominance.

In recent years histopathological examination requires the use of accessory diagnostic methods like immunohistochemistry which detects the

phenotype of tumors.It also helps in demonstrating the EBV virus association with lympho epithelial carcinoma in tissue sections.

Immunohistochemistry is also very helpful in distinguishing lymphoepthelial Carcinoma from lymphoma when there is diagnostic difficulty in histopathology.

AIMS AND OBJECTIVE

1. To study the prevalence of lymphoepithelial carcinoma

2. To study the role of immunohistochemical markers in the diagnosis of lymphoepithelial carcinoma

3. To differentiate lymphoepithelial carcinoma as a distinct entity among other carcinoma with intense lymphocytic infiltrations, using IHC markers and to treat accordingly

4. To demonstrate presence or absence of EBV antigen in lymphoepithelial neoplasms.

REVIEW OF LITERATURE

DEFINITION

Lymphoepithelial carcinoma is an undifferentiated malignant neoplasm arising from the nasopharyngeal mucosa that show evidence of squamous differentiation. The most common type of nasopharyngeal tumor is undifferentiated nasopharyngeal carcinoma, which is remarkable for the striking geographic differences in its incidence as well as the near consistent association with Epstein-Barr virus.

Nasopharyngeal carcinoma is also the prototype of a family of morphologically distinctive tumors – the lymphoepithelial carcinomas- that can arise in a variety of sites, head and neck mucosal sites, salivary gland, lung and thymus.

Interestingly, in contrast to nasopharyngeal carcinoma, lymphoepithelial like carcinomas occurring in these sites usually show a strong association with EBV only in Asians but not in Caucasions.[3]

SYNONYMS

- Lympho epithelioma
- Lympho epithelioma like carcinoma

- Undifferentiated carcinoma with lymphoid stoma
- Non keratinizing carcinoma
- Undifferentiated carcinoma

CLASSIFICATION OF NASOPHARYNGEAL CARCINOMA *In WHO Classification (1978)*

- Squamous cell carcinoma (WHO type1)
- Non keratinizing carcinoma (WHO type 2)
- Undifferentiated carcinoma (WHO type 3)

In WHO Classification (1991)

- Squamaous cell carcinoma
- Non Keratinizing carcinoma
- Differentiated carcinoma
- Undifferentiated carcinoma

Lympho epithelioma like carcinoma was considered a morphologic variant

of undifferentiated carcinoma[4]. Use of numerical designation of WHO types 1,2 and 3 was eliminated. The current WHO classification, maintains the terminology of the 1991 classification, with the addition of category. -Basaloid squamous cell carcinoma.

The term lymphoepithelioma was introduced in 1921 by Regaud of France and Schmincke of Germany to refer the undifferentiated carcinoma with an intense lymphocyte component. Lymphoepithelioma is an undifferentiated carcinoma with intense lymphoid infiltrates which originally was described in nasopharynx[5,6].

Tumors with the same morphologic pattern as nasopharyngeal lymphoepitheliomas occurring outside nasopharynx are called lymphoepithelioma like carcinoma.

These tumors have been described in a variety of organs, including the stomach, lung, salivary gland, thymus, urinary bladder, breast, esophagus, other head and neck mucosal sites, vagina, renal pelvis, thyroid gland, skin and lung[7,12]

EPIDEMIOLOGY

GLOBAL INCIDENCE AND MORTALITY

LEC constitutes 0.6% of all cancers. Incidence is higher in native and foreign born Chinese, South East African and North Africans. Highest incidence of NPC has long been observed in Hong Kong, where 1 in 40 men develop NPC before the age of 75 years[13].

AGE AND SEX DISTRIBUTION

In higher risk groups, LEC incidence rises after the age of 30 years and peak at 40-60 years and there after declines . Age distribution is similar in males and females, although it is seen 2-3 folds higher in men than in women[14].

ETIOLOGY

* Inter play of genetic susceptibility
* Infection by Epstein- Barr virus
* Environmental factors (dietary and non dietary) in disease causation

ESPTEIN- BARR VIRUS IN NASOPHARYNGEAL CARCINOMA

Near constant association of EBV with NPC, irrespective of ethnic

background, indicates a probable oncogenic role of the virus in the generation of this tumor[14]. EBV is a member of the Sub family of herpes virus, a group of Lymphotrophic viruses that includes human herpes virus 8.

GENES RELEVANT IN TRANSFORMATION AND REPLICATION

- Latent membrane proteins (LMP 1, LMP 2 A & LMP 2B)
- EBV nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA 3B EBNA3C)
- EBV early RNAS (EBER 1 and 2)
- RNA originating from the Bann HIA site.

NPC was the first epithelial neoplasm to be linked with EBV,serological data suggested a correlation between increased EBV antibody titres and NPC[16]. Subsequently, the EBV genome was identified in tumor extracts[15]. Nearly 100% of non keratinizing NPC are positive by Insitu Hybridization for EBV encoded EBER, regardless of patient ethnicity or geographic location. Evidence of oncogenic role of the virus in the genesis of the tumor includes,

1.Raised levels of antibodies especially IgA against EBV in most patients with NPC compared with normal controls.

2.Higher titres of IgA antibodies against EBV in patient with large tumor bulk.

3.Presence of EBV DNA or RNA in practically all tumor cells.

4.Presence of EBV in a clonal episomal form, indicating that the virus has entered the tumor cell before clonal expansion.

5.Presence of EBV in the precursor lesion of NPC but not in the normal nasopharyngeal epithelium.

The evidence was considered sufficient to classify EBV as carcinogenic by the international Agency for Research on Cancer (1ARC) in 1997[17]. Increased circulating levels of EBV DNA are associated with a worse prognosis and active disease and may provide a method for assessing patient risk and monitoring treatment response.

ENVIRONMENTAL FACTORS

Diet

- Occurrence of NPC is higher in regions were, high levels of volatile nitrosamines are used in preserved food [18].

- Preserved or fermented food and Consumption of salted fish[19] are seen to be associated with higher incidence

OCCUPATIONAL EXPOSURE

* Exposure to smoke, chemical fumes and dusts,

* Formaldehyde exposure,

* Prior radiation exposure, also are seen to be associated with higher risk of nasopharyngeal carcinoma.

LOCALIZATION

Most common site of origin is the lateral wall of the nasopharynx especially the fossa of Rosenmuller, followed by the superior posterior wall. Other sites include head and neck mucosa, salivary gland, lung, thymus, uterus, urinary bladder, and esophagus .

COMMON PRESENTING SYMPTOMS AND SIGNS OF NASOPHARYNGEAL CARCINOMA[20]

Presenting Features Symptoms	FREQUENCY (%)
Neck Mass	42%
Nasal (Post nasal drip, discharge, bleeding, obstruction)	46%
Aural (tinnitus, discharge, ear ache, deafness)	42%
Head ache	16%
Ophthalmic (double vision, squint, blindness)	6%
Facial numbness	5%
Speech/swallowing problem	2%
Weight Loss	4%

PHYSICAL SIGNS

PRESENTING FEATURES	FREQUENCY (%)
Enlarged neck nodes	72%
Bilateral neck nodes	35%
Neck nodes extending to supra clavicular fossa	12%
Cranial nerve palsy	10%
Deafness	3%
Dermatomyositis	1%

IMAGING

Magnetic Resonance (MRI) is the study of choice for assessing the loco-regional extent and intra cranial extension. Computerized Tomography is useful in depicting cortical bone erosion.

SEROLOGICAL STUDIES

Positive serology against Epstein-Barr virus is found in close to 100% of patients with non-keratinizing NPC[21].Detection of 1gA against viral capsid antigen (VCA) and 1gG/1gA against early antigens (EA) are the most extensively used diagnostic tool.

Newer antibody tests based on Recombinant EBV antigens such as EB nuclear antigen (EBNA), membraneantigen (MA), thymidinekinase (TK), DNApolymerase, DNAse and Z Tran activator protein (Z at) can be used in combination,

RELEVANT DIAGNOSTIC PROCEDURES

- Complete physical examination and endoscopic examination of the nasopharyngeal region.

- Biopsies taken from the gross lesions.

- In the absence of a gross lesion multiple biopsies should be taken from the lateral. superior and posterior walls of nasopharynx for patients with high suspicion of NPC.

MACROSCOPY

The tumor can appear as a smooth bulge in the mucosa ,a discrete raised nodule with or without surface ulceration or a frankly infiltrative fungating mass. Some times no grossly visible lesion is seen.

PATTERNS OF SPREAD

Primary

Inferior extension along the lateral pharyngeal walls and tonsillar pillars occur in almost one third of patients. Extension into the posterior nasal cavity is frequent but usually limited to less than 1 cm. Invasion of the Posterior ethmoids, the maxillary antrum, and the orbit occurs fairly often. Invasion into or through the skull base is recognized radiographically.In at least 25% of patients the sphenoid sinus is frequently involved. Tumor may erode through the foreman ovale, the foramen lacerum and the foramen spinosum. Tumor eventually reaches the cavernous sinus and has access to cranial nerves II and III.

LYMPHATIC SPREAD

There is an 80% to 90% incidence of metastatic neck node disease on presentation; approximately 50% of patients have bilateral lymphnode metastasis. NPC often metastasis to level V lymphnodes, Midline tumors often spread bilaterally.

HISTOPATHOLOGY

The tumor comprises of solid sheets, irregular islands, dyscohesive sheets and trabeculae of carcinoma cells intimately intermingled with variable numbers of lymphocytes and plasma cells. Two patterns of growth may be

seen, some times in combination The first, referred to as Regaud's type, consists of well defined aggregates of epithelial cells surrounded by fibrous tissue and lymphoid cells.[22] In the second, designated as Schmincke's type, the neoplastic epithelial cells grow diffusely and are closely intermingled with inflammatory cells. The nuclei of NPC tend to be vesicular with a smooth outline and a single, large, eosinophilic nucleolus.

CRITERIA FOR DIAGNOSING LYMPHOEPITHELIAL CARCINOMA OF NASOPHARNGEAL ORIGIN[23]

1.Primary untreated tumor should be of poorly differentiated carcinoma without keratin pearls, intra cellular bridges or individual cell keratinization.

2.Must exhibit either Schmincke or Regaud type of patterns and should be keratin positive by immunohistochemistry.

3.Tumor should be centered either in the region of the fossa of Rosenmuller or on the vault or posterior wall of the nasopharynx.

EB VIRUS STATUS

4.Positivity for EB virus antibody titres which should be raised or if it is normal EB virus moieties must be demonstrated in the tumor cells by Insitu

Hybridization for EB virus – encoded RNAS.

LYMPHOEPITHELIAL CARCINOMA OUTSIDE NASOPHARYNX

The histology of lymphoepithelial carcinoma outside nasopharynx is the same as that of lymphoepethelial carcinoma of the nasopharynx. EBV association is less or lacking in most of the sites other than nasopharynx. However, EBV said to be constantly associated with lymphoepethelial carcinoma in 3 anatomical sites out side the nasopharynx, stomach, salivary gland and lung.

IMMUNO PROFILE IN LYMPHOEPITHELIAL CARCINOMA

Practically all tumor cells show strong staining for Pan-cytokeratin (AE1/AE3), MNF – 116). The staining for high molecular weight cytokeratins (Such as cytokeratins 5/6, 34 βe 12) is strong and staining for low molecular weight cytokeratins such as (CAM 5.2) in often weaker and sometimes patchy.

Cytokeratins 7 and 20 are both negative[24]. In differentiated non keratinizing carcinoma the cytokeratin immunostain highlights the scanty wisps of cytoplasm that wrap around the large nucleus and extend outward as short narrow processes. As a result of the cell nests being broken up by infiltrating lymphocytes, a distinctive reticulated or meshwork pattern is produced[25,26, 27, 28, 29].

Immuno reactivity for epithelial membrane antigen in nasophangeal carcinoma is often only focal. In most cases, the tumor exhibits strong nuclear staining for p63, a basal cell marker that mainly highlights the basal and parabasal cells of the overlying stratified squamous epithelium. The lymphoid cells represent a mixture of T cell and B cells, usually with the former predominating especially within and around the tumor islands. At least a proportion of the T cells are activated cytotoxic T cells. The plasma cells are polyclonal.

There are variable numbers of scattered S100 protein positive dendritic cells. Some studies have reported the following features to be associated with a better prognosis.

- High density of dendritic cells
- High number of infiltrating lymphocytes
- Fewer number of granzyme B- positive cytotoxic cells.

EPSTEIN – BARR VIRUS DETECTION

Lymphoepithelial carcinoma of nasopharynx is associated with Epstein Barr virus in practically 100%, of cases, irrespective of the ethnic background of the patient.

Latently infected B cells express a limited number of EBV genes; 6 nuclear protein (EBNA-1, EBNA-2, EBNA-34, EBNA-3B, EBNA-3C and

EBNA – LP),

3 membrane proteins (LMP:-1, LMP-2A, and LMP-2B), EBV encoded small RNAS (EBER 1 and EBER 2) and RNA transcripts from the Bam HIA region.

Type I Latency: is characterized by expression of EBNA – 1 as well as the RNA transcripts for EBER 1, EBER 2 and the Bam HIA region. The presence of EBNA 1 is the minimal expression state since this protein is required for episomal replication and in its absence, the virus is not propagated. This type of latency has not been identified in either epithelial or mesenchymal neoplasms.

In type II latency, LMP 1 and LMP 2 are expressed in addition to those of type I latency.

Type II latency is seen in EBV – associated Hodgkin's lymphoma, peripheral T/NK cell lymphoma and undifferentiated NPC[30,31]. LMP 1 is an integral transmembrane protein that functions as a constitutively active member of the tumor necrosis factor receptor family.

LMP 1 potentiates a variety of signaling pathways including the nuclear factor κB, mitogen activated protein kinase, and phophotidylinositol 3-Kinase Alt Pathways[32] and involves in angiogenesis which is the key step in tumor growth, invasion and metastasis.

LMP 1 protein is essential for EBV – mediated immortalization of B lymphocytes[33] and its expression has been shown to inhibit the terminal differentiation of keratinocytes, thus providing a possible explanation for the lack of differentiation of most EBV associated epithelial tumor cells.

In addition to oncogenesis, LMP-1 is suggested to be relevant to the metastatic property of NPC[34,35&36]. Studies also report that LMP-1 positive NPCs show a more progressive attitude and an increased tendency towards lymph node metastasis than LMP-1 negative NPCs. Recent study[71]. suggests that induction of c-met proto-oncogene by LMP-1 in mediated by activation of Ets-1 transcription factor which leads to, upregulation of cell motility considered to be an essential factor in the multiple steps of metastasis.

To confirm, the association of EBV in a given tumor, the virus must be detected with in the tumor cells. EBV latent membrane protein-1 (LMP-1) is usually positive in only 30-40% cases[37,38] and the immuno staining is often patchy. How ever one recent study[39] states that no biopsy is completely devoid of LMP-1 positive cells and also suggests the use of S12 antibody which is more sensitive in staining tissue sections than CS1-4 antibody.

The nested PCR technique is one of the most powerful methods for identifying specific DNA fragments. The simplest and most reliable way to demonstrate EBV is Insitu hybridization for EBV encoded early RNA (EBER) which is present in abundance in cells latently infected by EBV[40-46].

2002 AMERICAN JOINT COMMITTEE ON CANCER STAGING FOR NASOPHARYNGEAL CANCER

Primary Tumor (T)

T_1	:	Tumor confined to the nasopharynx
T_2	:	Tumor extends to soft tissues.
T_{2a}	:	Tumor extends to the oropharynx and/or Nasal cavity without parapharyngeal extension.
T_{2b}	:	Any tumor with parapharyngeal extension.
T_3	:	Tumor involves bony structures and/or paranasal sinuses
T_4	:	Tumor with intra cranial extension, infra temporal fossa, hypopharynx, orbit or masticator space.

REGIONAL LYMPHNODES (N)

N_X		Regional lymphnodes cannot be assessed.
N_0		No regional lymphnode metastasis
N_1		Unilateral metastasis in lymphnode (S) 6cm or less in greatest dimension, above the supraclavicular fossa
N_2		Bilateral metastasis in lymphnode (S) 6cm or less in greatest dimension, above the supraclavicular fossa.
N_{3a}		Metastasis in a lymphonode (S) greater than 6 cm
N_{3b}		Extension to the supraclavicular fossa.

STAGE GROUPING

O	Tis	No	Mo

I	T_1	No	Mo
II A	T_{2G}	No	Mo
II B	T_1	N_1	Mo
	T_{2a}	N_1	Mo
	T_{2b}	$N_0 - N_1$	Mo
2III	T_1	N_2	Mo
	$T_{2a} - T_{2b}$	N_2	Mo
	T_3	$N_0 - N_2$	Mo
IV A	T_4	$N_0 - N_2$	Mo
IV B	Any T	N_3	Mo
IV C	Any T	Any N	M_1

PROGNOSIS

The presenting stage is the most important prognostic factor. 5 Year Disease specific survival for stage I is 98%. Stage II A-B 95% stage III 86% and stage IV A-B 73%.

Younger age and female patients are associated with better prognosis[47]. Tumor volume may prove to be useful for predicting local control. Circulating plasma/serum EBV DNA is a more promising prognostic factor.

Higher titers are associated with advanced stages[48]. High density of dendritic cell, high number of infiltrating lymphocytes and low number of granzyme B-Positive cytotoxic cells are associated with better prognosis[25,29]. Aneuploid status or high pretreatment tumor proliferative fraction, co-rrelate significantly with poor survival.

IMMUNOHISTOCHEMISTRY

GENERAL CONCEPTS OF IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a method for localizing specific antigens in tissues or cells based on antigen – antibody recognition. IHC has a long history, extending to more than half a Century from 1940 when Coons developed an Immunofluoresence technique to detect corresponding antigen in frozen sections[49]. However only since the early 1990 has the method found general application in surgical pathology.

A series of technical developments in IHC have created sensitive detection systems. Among them is the enzymatic label (Horse radish peroxidase) developed by Avrameas and Colleagues[50] which in the presence of suitable colorigenic substrate systems, allowed visualization of the labeled antibody by light microscopy.

One of the critical issues in the development of immuno peroxidase techniques was related to the need to achieve greater sensitivity from the simplest one step direct conjugate method to multi step detection techniques such as the peroxidase – Antiperoxidase, Avidin- biotin Conjugate and Biotin Streptavidin methods, together with amplification methods and highly sensitive polymer based labeling systems[51].

The development of the Hybridoma technique facilitated the

development of IHC and the manufacture of abundant highly specific monoclonal antibodies, many of which found early application in staining of tissues, Only when the IHC became applicable to routinely formalin fixed, paraffin embedded tissue sections did it bring in the “brown revolution”. The critical significance of rendering the IHC technique suitable for routine paraffin sections was illustrated by Taylor and colleagues, who in 1974, showed that it was possible to demonstrate antigens in routinely processed tissue.

Enzyme digestion was introduced by Huang and colleagues as a pretreatment to IHC staining to unmask some antigens that had been altered by formalin fixation. However the enzyme digestion method, while widely applied did not improve IHC staining of the majority of antigens as perceived by Leong and colleagues.

Another drawback of enzymes digestion was that it has proved difficult to control the optional digestion condition for individual tissue sections when stained with different antibodies. The difficulties in standardization provided a powerful incentive for the development of a new technique.

The Antigen retrieval (AR) technique[52] was developed by Shi and associates in 1991. In contrast to enzyme digestion the AR technique is a simple method that involves heating routinely processed paraffin sections at high temperatures before staining procedures. The intensity of IHC staining was increased dramatically after AR Pretreatment, as demonstrated by various articles[53].

BASIC PRINCIPLES OF IMMUNOHISTOCHEMISTRY

The object of all stains is to recognize micro chemically the existence and distribution of substances which we have been made aware of macro chemically. The basic principle of IHC as with any other special staining method is a sharp localization of target components in the cell and tissue based on a significant signal – to – noise ratio. Amplifying the signal, while reducing the non specific background staining (noise) is the major strategy to achieve a satisfactory and practically useful result.

An antibody is a molecule that has the property of combining specifically with a second molecule, termed the antigen. Antigen-antibody recognition is based on the three dimensional structure of protein or antigen, which is a critical issue in the understanding of the effectiveness of IHC as well as the mechanisms of AR.

The term epitope corresponds to a cluster of aminoacid residues that bind specifically to the paratope of an antibody. An epitope is a functional unit and not the structural element of a protein and may be classified as continuous and discontinuous. The former are compounds, of a continuum of residues in a polypeptide chain, where as the latter consist of residues from different parts of a polypeptide chain, brought together by the folding of the protein conformation.

The development of the Hybridoma technique provided an almost

unlimited source of highly specific antibodies, although monoclonal antibodies do not guarantee antigen specificity, Different antigens may share similar or cross reactive epitopes, and the practical specificity reflected by IHC is excellent for most monoclonal antibodies tested.

In contrast a polyclonal antibody is in fact an antiserum, which contains several different molecular species of antibody having varying affinities and even varying specificities against the different antigens or antigenic determinants. As a result polyclonal antibodies may give the most non specific background staining in slides than the staining obtained using monoclonal antibodies.

Comparison of sensitivity and specificities between polyclonal and monoclonal antibodies indicate that polyclonal antibody may be more sensitive but less specific than monoclonal antibody. The reason may be that polyclonal antibody may recognize several different epitope on a single antigen whereas a monoclonal antibody recognizes only a single type of epitope.Sophisticated amplification techniques, coupled with use of the AR technique have reduced the practical importance of this distinction.

Although the specificity of monoclonal antibody has been questioned regarding cross reactivity with non-target molecules, most community available monoclonal antibodies are highly reliable for IHC, but again the

ultimate specificity control should be the observation of the expected pattern of staining in control tissue sections, with the corresponding lack of unexpected or inexplicable staining reactions[53, 54].

TISSUE FIXATION, PROCESSING AND ANTIGEN RETRIEVAL TECHNIQUES

The ideal fixative for IHC studies should not only be readily available but should also be in widespread use to maximize the range and number of samples available for IHC studies. The fixative should preserve antigen integrity and should limit extraction, diffusion or displacement of antigen during subsequent processing. It should also give good preservation of morphological details after embedding in a supporting medium.

Common fixatives used in Histopathology are divided into two groups, coagulative fixatives such as ethanol and cross linking fixatives such as formaldehyde. Both types of fixatives cause changes in the stearic configuration of proteins which may mask epitopes and adversely affect bonding with antibody.

In most surgical pathology laboratories the fixative used is 10% neutral buffered formalin. Traditionally non cross linking fixatives are believed to be superior to aldehyde fixation in order to retain immuno reactivity for certain

larger protein such as intermediate filaments and immunoglobin.

ADDITIONAL ADVANTAGES OF FORMALIN INCLUDE

1. Good morphological preservation.
2. Cheap.
3. Sterilizes tissues.
4. Carbohydrate antigens are better preserved.
5. Many antigens are preserved during the process of cross linking.

Formalin may be regarded as a satisfactory fixative for both morphology and IHC provided that a simple and effective AR technique is available to recover those antigens that are diminished or modified.

Subsequent treatment with absolute ethanol during dehydration serves as double fixation.

ANTIGEN RETRIEVAL

The disadvantage of masking of antigens during fixation can be overcome by antigen retrieval technique. Process involves unmasking of antigens by one of these four techniques:

1. Proteolyte enzyme digestion

2. Microwave antigen retrieval

3. Microwave and trypsin antigen retrieval technique.

4. Pressure Cooker antigen retrieval.

Trypsin enzyme digestion is difficult to control**.**

A Simple heat induced AR technique is now widely applied in pathology. Successful application of the AR technique for routine IHC Staining of formalin fixed tissue has rendered the search for alternative fixatives to replace formalin less urgent. In AR technique involving heat, the result is influenced by heating condition (temperature) and time of heating) and P^H of the AR Solution. High temperature is the most important factor.

Microwave AR technique is a new technique. Heating is done in plastic coplin jars. Drying of sections can take place and hence careful monitoring is

required. Pressure cookers do not require close inspection and do not suffer from inconsistent results. In any of the conditions where in heat is employed slides are coated with silane to prevent loss of sections.

Among the Various solutions, citrate buffer at pH 6.0, EDTA at pH 8.0 and TRIS- EDTA at PH 9.9 or 10.0 are most popular

Both microwaves and pressure cookers with or without additional enclosures for temperature control and moisture are used successfully for retrieval of antigens like cytokeratin[55].

MATERIAL AND METHODS

The study was conducted from the period of 2006-2009 both prospectively & retrospectively. All the biopsy samples received in the Department of Pathology, Stanley Medical College, were analysed. Total of 57 cases with malignant lesions showing prominent lymphoepitheial component were taken for the study. All samples taken from nasopharynx were given special reference.

Epithelial malignances showing rich lymphoplasmacytic infiltrations were taken for analysis. Samples routinely processed and stained with H&E were analysed followed by IHC markers. Epithelial and lymphoma markers were used to confirm the histopathological diagnosis of lymphoepithelial carcinoma.

LMP-1 immunohistochemical marker was used to demonstrate EBV in tissue sections. Lymphoepitheial carcinoma reported at Kilpauk Medical College, Madras Medical College, and Government Royapettah Hospital were also included for the study purpose.

INCLUSION CRITERIA

Tumors which were diagnosed as lymphoepithelial carcinoma/lymphoma and poorly differentiated carcinoma with lymphoid aggregates were included.

EXCLUSION CRITERIA

Tumors which were diagnosed histopathologically as well differentiated and moderately differentiated squamous cell carcinoma and other benign tumors were excluded.

METHOD OF DATA COLLECTION

The material consisted of 57 cases whose clinical details were obtained. Out of 57 cases, 34 were lymphoepithelial carcinoma, 21cases were poorly differentiated carcinoma, 2 cases had doubtful histological diagnosis and reported as lymphoepithelial carcinoma/lymphoma for which IHC was done for confirmation.

METHOD OF TISSUE PREPARATION FOR IHC

10% buffered neutral formalin were used for fixation of specimens. The tissues were processed in automated histokinette through various grades of alcohol and xylol. Paraffin blocks were prepared; Sections were cut using Semi automatic Microtome with disposable blades and stained with hemotoxylin and eosin .Suitable sections were chosen for IHC.

Slides wee coated with chrome alum, and subjected to AR using the Microwave technique with TRIS –EDTA buffer solution .Slides were then treated by HRP (Horse radish peroxidase) polymer technique.

HRP POLYMER TECHNIQUE

The coated slides were taken through the following steps

1. Treatment with peroxidase block – for incubation of endogenous peroxidase in the tissue for 20 minutes, washed in TRIS buffer for 5 minutes.

2. Application of power block O- to block non specific antigen – antibody reactions for 20 minutes. The excess power block was blot dried.

3. Application of Primary antibody – Murine antibodies for 60 minutes. Washed in TRIS buffer for 5 minutes.

4. Application of super enhancer for 30 minutes which increased the sensitivity of antigen -antibody reaction thereby enhancing the final reaction product.

5. Application of SS label – Secondary antibody from goat with the tagged horse radish peroxidase enzyme for 30 minutes. Washed in TRIS buffer.

6. Application of DAB (Diamino benzidine) Chromogen for 5 minutes – which was cleared by the enzyme to give the colored product at antigen sites. Washed in distilled water for 5 minutes.

7. The slides were then counter stained with hematoxylin .Slides were air dried and mounted with DPX (Distrene dibutyl pthalide in Xylol)

IHC MARKERS USED

PHENOTYPIC MARKERS

Pan cytokeratin and leukocyte common antigen were used for confirming the histopathological diagnosis in all undifferentiated carcinoma. CD20, CD3 were additionally used in doubtful cases to rule out lymphoma.

SPECIFIC MARKER

Latent membrane protein -1 (CS1-4 antibody) was used in all cytokeratin positive cases. Section of Hodgkin lymphoma was taken as control for LMP-1 (Fig 21,22).Immuno-staining was scored on the basis of positive tumor cells and the relative immunostaining intensity.Five consecutive microscopic fields were analyzed.

The following grading system was adapted to score the number of positive cells:

Scoring method[39]

- 0-none seen in the section
- 1-presence of rare positive cells but not exceeding 25%
- 2-26 to 50% positive cells
- 3-51 to 75% positive cells
- 4-76 to 100% positive cells

IMMUNOSTAINING INTENSITY

- 0-none
- 1-weak
- 2-moderate
- 3-intense

OBSERVATION AND RESULTS

Out of 57 biopsy specimens which were diagnosed as lymphoepithelial carcinoma/ lymphoma were taken for the study.

These included,

- 34 cases of undifferentiated carcinoma (Fig 1-8)
- 21 cases of poorly differentiated carcinoma
- 2 cases whose histopathological diagnoses were doubtful and needed immunohistochemistry for confirmation.

Immunohistochemistry was done using pancytokeratin and leukocyte common antigen on 34 cases of undifferentiated carcinoma to confirm the histopathological diagnosis.33 cases were found to be positive for pancytokeratin. (Fig 12,13,17) and CD 45, CD20,CD3 were found to positive in infiltrating lymphocytes. (Fig 14,15,16,18,19 and 20)

25 cases of pancytokeratin positive undifferentiated carcinoma were selected for latent membrane protein -1 immunostaining for detecting EBV association

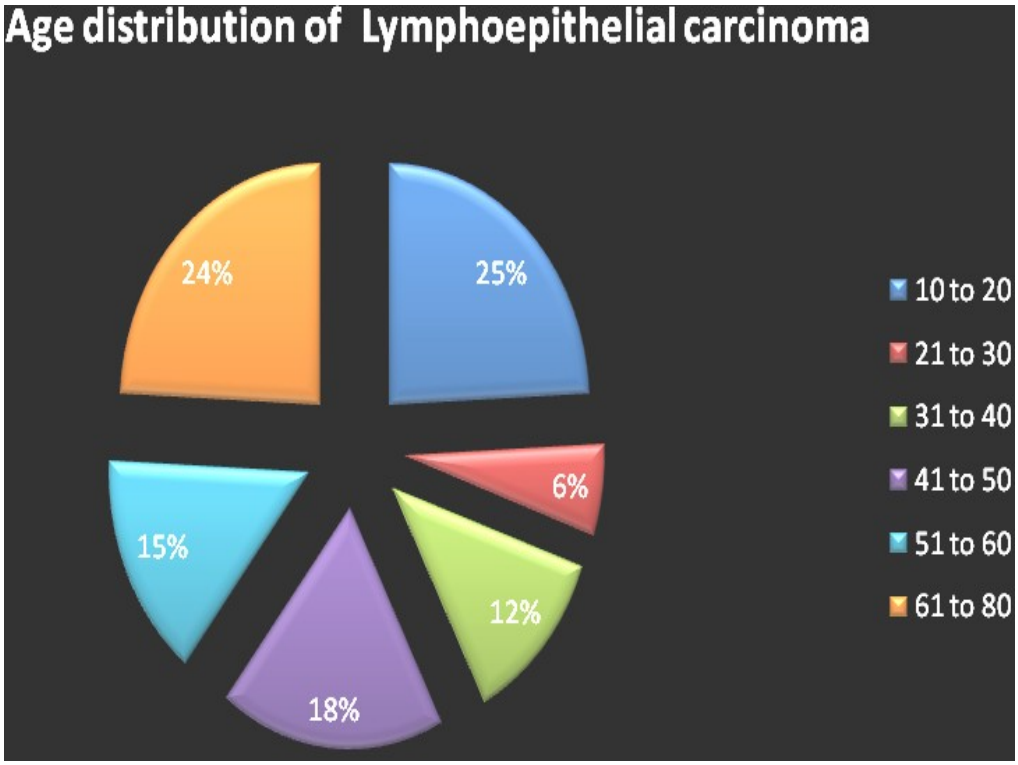
AGE DISTRIBUTION OF LYMPHOEPITHELIAL CARCINOMA

TABLE - 1

Years	No of cases
10 to 20	9
21 to 30	2
31 to 40	4
41 to 50	6
51 to 60	5
61 to 80	8
Total	34

Majority of cases belonged to younger age group (10-20) and 61-80 years of age showing a bimodal distribution.

AGE DISTRIBUTION OF LYMPHOEPITHELIAL CARCINOMA



SEX DISTRIBUTION

Out of 34 cases 23 were male patients and 11 were female patients.

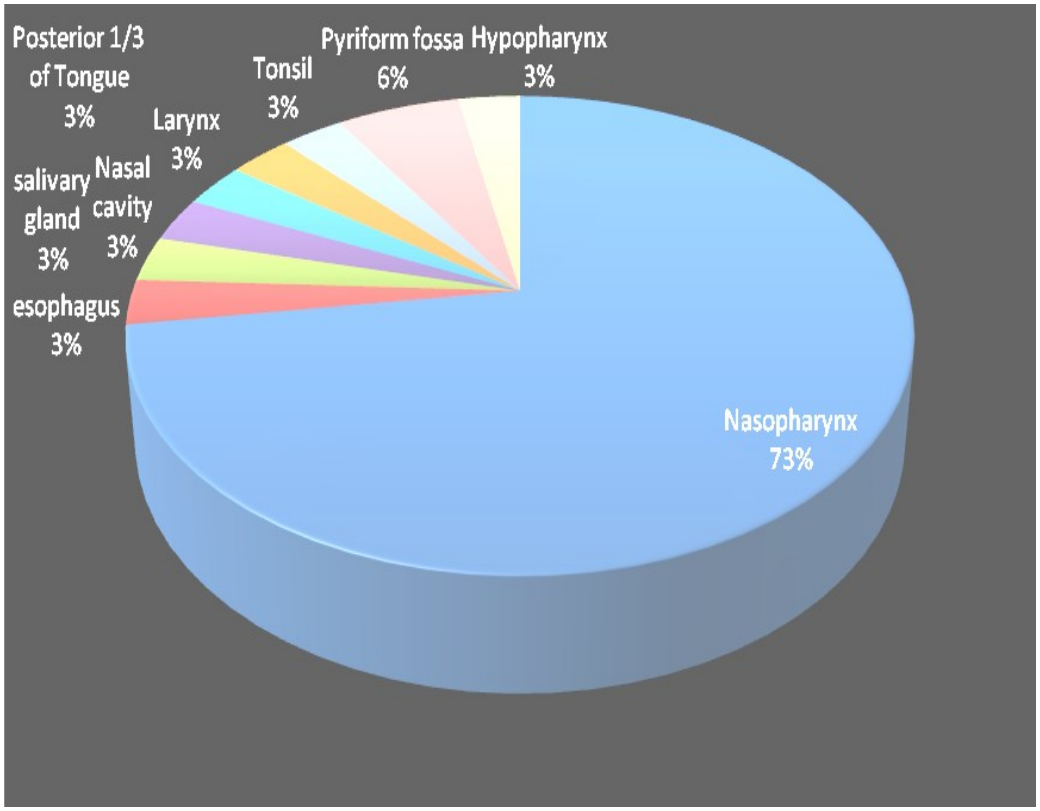
SITE DISTRIBUTION OF CASES

Majority of cases (24) were found in the nasopharynx, 2 cases were in pyriform fossa, 2 cases were in posterior one third of tongue. Rest of the case were found in Nasal cavity (1) tonsil (1), hypopharynx (1), epiglottis (1), esophagus (1), salivary gland (1), larynx (1).

TABLE - 2

Site	No of cases
Nasopharynx	24
Esophagus	1
Salivary gland	1
Nasal cavity	1
Larynx	1
Posterior 1/3 of Tongue	1
Tonsil	1
Pyriform fossa	2
Hypopharynx	1
Epiglottis	1
Total	34

SITE DISTRIBUTION OF CASES



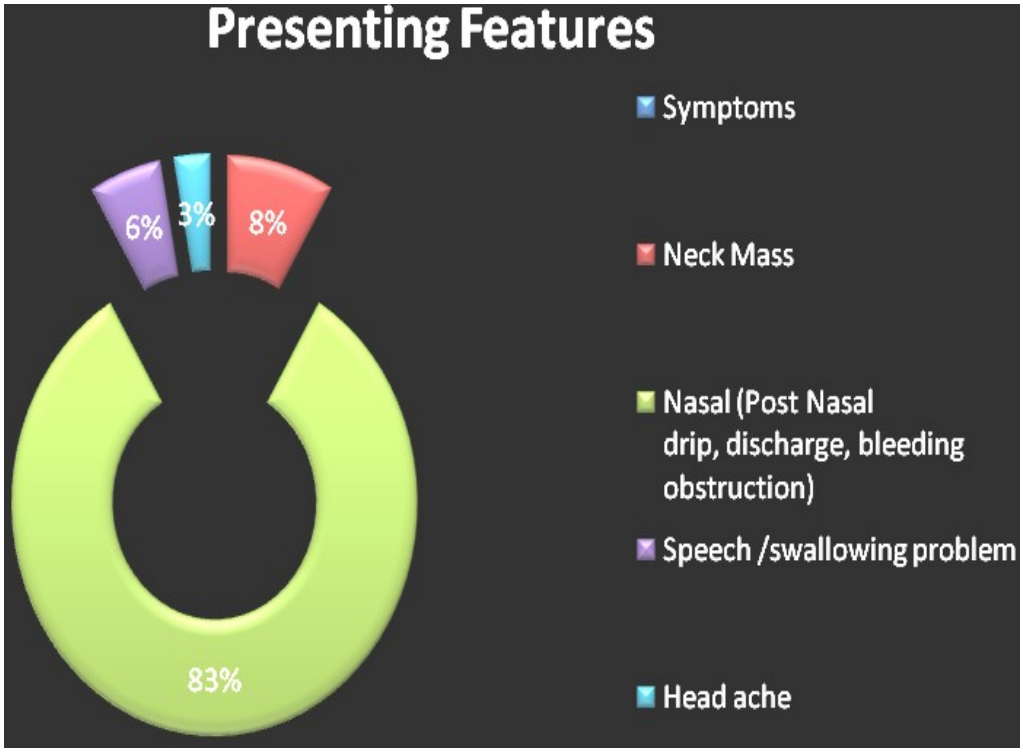
CLINICAL PRESENTATION

Most of the cases presented with nasal symptoms like nasal obstruction,discharge per nose (29 cases) . 3 cases presented with cervical lymphnode as the initial manifestation,for which FNAC was done and reported as LEC/lymphoma.2 cases presented with swallowing/speech problem

TABLE - 3

Presenting features - Symptoms	**Frequency**
Neck Mass	8%
Nasal (Post Nasal drip, discharge, bleeding obstruction)	83%
Speech /swallowing problem	6%
Head ache	3%

CLINICAL PRESENTATION



IMMUNOHISTICHEMISTRY RESULTS

TABLE - 4

S. No	Biopsy No.	Site	HPE	CK	Lymphoma Panal	LMP-1
1	7281/06	nasopharynx	LEC	+	focal	intense
2	7545/06	nasopharynx	LEC	negative	negative	
3	815/07	Esophagus	LEC	+	focal	negative
4	983/07	Post.1/3 tongue	LEC	+	focal	moderate
5	1607/07	nasopharynx	LEC	+	focal	weak
6	516/08	nasopharynx	LEC	+	focal	weak
7	731/08	Pyriform fossa	LEC	+	focal	weak
8	1356/08	nasopharynx	LEC	+	Focal	intense
9	1438/08	nasopharynx	LEC	+	focal	weak
10	2740/08	hypopharynx	LEC	+	focal	negative
11	2743/08	nasopharynx	LEC	+	focal	Weak
12	2774/08	nasopharynx	LEC	+	focal	intense
13	2890/08	nasopharynx	LEC	+	focal	Moderate
14	3085/08	nasopharynx	LEC	+	focal	moderate
15	3553/08	epiglottis	LEC	+	focal	negative
16	4598/08	Salivary gland	LEC	+	focal	intense
17	4621/08	stomach	LEC /poorly Differentiated Carcinoma	+	diffuse	negative
18	4825/08	Soft palate	LEC	+	focal	negative
19	5055/08	tonsil	LEC/lymphoma	negative	positive	-
20	5063/08	larynx	LEC	+	focal	negative
21	8414/08	nasopharynx	LEC	+	focal	moderate
22	101/09	nasopharynx	LEC	+	focal	moderate
23	2789/09	tonsil	LEC	+	focal	negative
24	2834/09	nasopharynx	LEC	+	focal	moderate
25	4939/09	nasopharynx	LEC	+	focal	moderate
	4939/09	Cervical node	Metastatic/lymphoma	negative	positive	negative

Latent membrane protein -1 expression in tumor cells Immunohistochemistry using the anti LMP-1 antibody resulted in highly heterogeneous staining between tumors from different patients. It was assessed using a scoring system based on the percentage of positive cells and the intensity of staining. (Fig 22, 23, 25, 26, 27, 28)

SCORING METHOD

- 0-none seen in the section
- 1-presence of rare positive cells but not exceeding 25%
- 2-26 to 50% positive cells
- 3-51 to 75% positive cells
- 4-76 to 100% positive cells

IMMUNOSTAINING INTENSITY

- 0-none
- 1-weak
- 2-moderate
- 3-intense

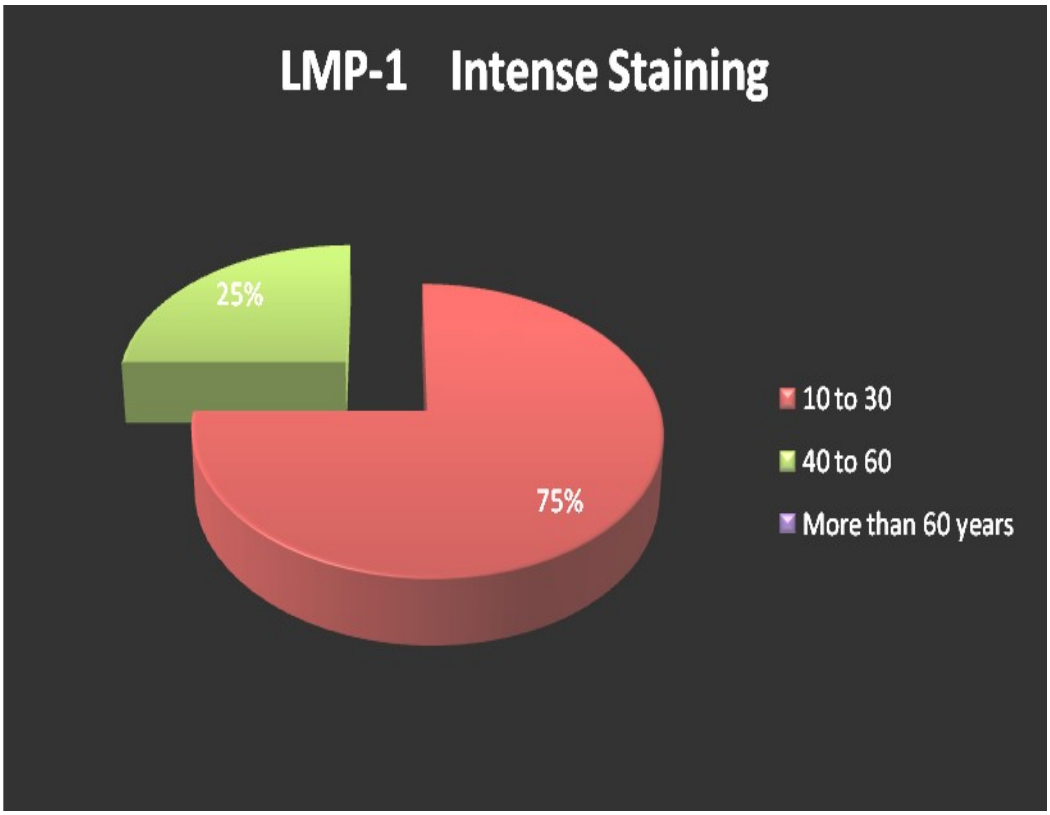
CLINICAL CORRELATION OF LMP-1 WITH AGE OF THE PATIENTS

TABLE - 5

Age	LMP-1		
	Intense	**moderate**	**weak**
10 to 30	3	2	1
40 to 60	1	2	3
More than 60 years	-	1	-

Younger age group showed higher levels of expression.

CLINICAL CORRELATION OF LMP-1 WITH AGE OF THE PATIENTS



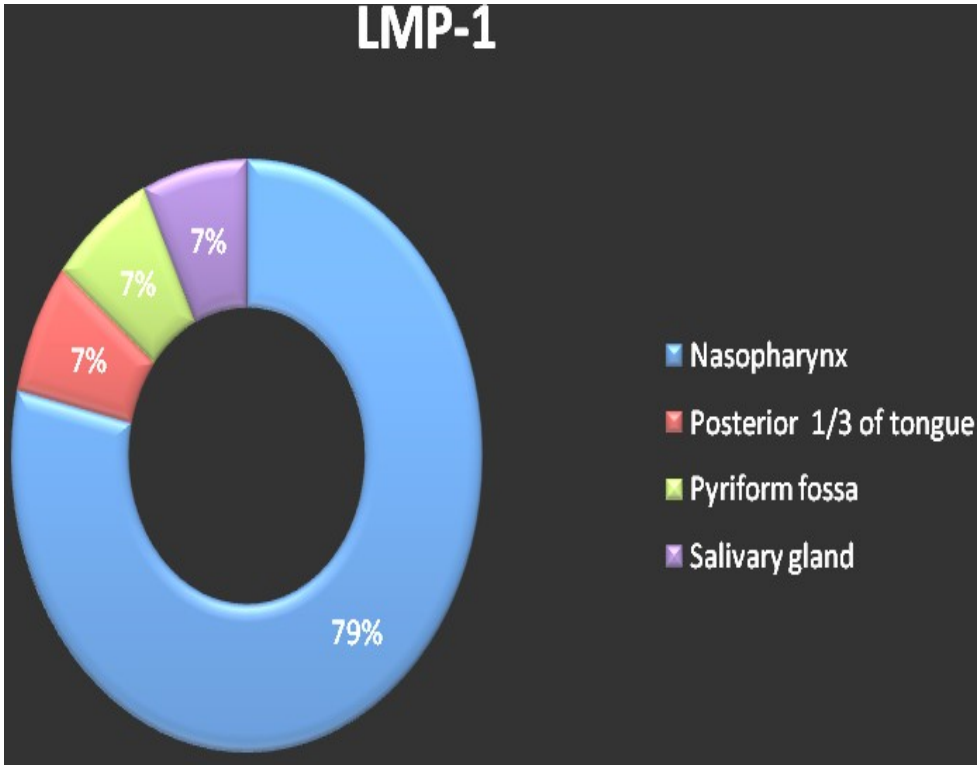
CLINICAL CORRELATION OF LMP-1 WITH SITE OF OCCURRENCE

LEC from nasopharynx showed strong expression of LMP-1 when compared to LEC from other sites except salivary gland.

TABLE - 6

Site	LMP-1
Nasopharynx	13
Posterior 1/3 of tongue	1
Pyriform fossa	1
Salivary gland	1

CLINICAL CORRELATION OF LMP-1 WITH SITE OF OCCURRENCE



TUMORS IN WHICH IMMUNOHISTOCHEMISTRY HELPED IN CONFIRMING THE DIAGNOSIS

CASE 1

Excision specimen of a laryngeal mass which presented with obstructive symptoms in a 60 year old male was received . Histopathological examination revealed a neoplasm arranged in diffuse sheets with interspersed lymphocytes. High power view showed pleomorphic cells with vesicular nuclei. Some of them showed prominent nucleoli. (Fig 37)

Considering the differential diagnosis of LEC and lymphoma, IHC was done using pan-cytokeratin and lymphoma panel .CD 45, CD20 (Fig 38) was found to be positive and CD3 and pan cytokeratin were negative.

With IHC showing CD45, CD20 positivity, this case was confirmed to be Large B cell lymphoma.

TABLE - 7

IHC markers	Results
Pancytokeratin	Negative
CD45	Positive
CD20	Positive
CD3	Negative

CASE - 2

Total gatrectomy specimen which presented abdominal fullness in a 60 year old female was received . Histopathologically the neoplasm showed sheets of poorly differentiated cells with dense lymphoid stroma.

IHC was done with pan cytokeratin ,and lymphoma panel .cytokeratin was positive in tumor cells and CD45,CD20,CD3 were positive in non neoplastic lymphocytes .This case was again stained with latent membrane protein -1, and it was found to be negative.

Studies showed that LEC of gastric mucosa was strongly associated with EBV virus[56,57]. Since LMP-1 was found to positive in only 30-40% of cases as per previous studies, further molecular studies are needed for EBV detection in this case.

TABLE - 8

IHC markers	Results
Pan cytokeratin	Positive
CD45	Positive in non neoplastic lymphocytes
CD20	Positive in non neoplastic lymphocytes
CD3	Positive in non neoplastic lymphocytes
LMP-1	Negative

CASE - 3

67 years old male patient presented with nasal obstruction and cervical neck node. ENT examination revealed a nasopharyngeal mass .Biopsy specimens from both the sites examined histopathologically.

Nasopharyngeal mass showed feature of undifferentiated carcinoma of nasopharynx (Fig 31, 32). IHC was done with pancytokeratin and lymphoma markers (CD45, CD20, CD3). Pan cytokeratin was found to be positive, (Fig 34) and CD45, CD20, CD3 were positive in non neoplastic lymphocytes.

Sections from cervical node revealed monotonous population of

lymphoid cells with out epithelial differentiation (Fig 35) showing completely different morphology in contrast to the primary nasopharyngeal mass. IHC was done, using pancytokeratin, CD45, CD20, CD3. CD45, CD20 (Fig 36) were positive in the lymphoid cells and pancytokeratin and CD3 were negative.

Both the sections were again stained with latent membrane protein-1 and it was found to be positive in only in nasopharyngeal mass (Fig 35) and negative in cervical node .Finally this case was reported as EBV associated lymphoepithelial carcinoma, with associated small lymphocytic lymphoma – cervical lymphnode .

TABLE - 9

IHC markers	**LEC**	**SLL**
Pancytokeratin	positive	negative
CD 45	Focal	Positive
CD 20	focal	Positive
CD 3	Focal	negative
LMP-1	positive	negative

DISCUSSION

Nasopharyngeal carcinoma is often difficult to diagnose because of nonspecific nature of its clinical symptoms and the difficulty in visualizing the nasopharynx[58]. Submucosal primary lesions often escape endoscopic examination(59).Most of the tumors remain undiagnosed until they present as metestasis to cervical lymph nodes,often without overt pathology at the primary site[60]. FNAC will be very much helpful in such cases[60,62]. One such case was diagnosed with FNAC ,in cervical node and it correlated with HPE diagnosis (Fig 9, 10) .

Koppiker et. al, have reported that 5 yr actuarial disease free survival of Indian patients is 13%[61] which indicates the need for an early diagnosis and treatment strategies to improve survival. Early detection of NPC should improve cure rate and reduce morbidity and metastasis. This requires an effective screening system.

Ethnic and regional factors are found to strongly influence the risk of disease. However ,there have been very few well conducted study on Indian patients[64]. The present study assess the age distribution, sex distribution , relationship between EBV and sporadic Indian NPC and the role of latent membrane protein -1 in NPC detection.

Out of 34 cases of lympho epithethelial carcinoma 9 cases were found in the younger age group (10 – 20) yrs and 8 cases in the older age group (61

-80) yrs Balakrishnan et.al[63] observed bimodal age distribution with peaks in age group 15-24 and 45-54 yrs from India.

This present study confirms the bimodal distribution except for the increase in peak in 60-80 yrs of age group in contrast to 45-54 age group in the study of Balakrishnan et. al . This study also confirms the male preponderance of lymphoepithelial carcinoma as in previous studies.

Most of the patients in this study, presented with nasal obstruction and nasal bleeding (83%). Three of them presented with cervical node metastasis as the initial manifestation.

Among 34 cases were undifferentiated carcinoma, 23 cases were found in nasopharynx, 2 cases in pyriform fossa. Rest of them were found in hypopharynx , nasal cavity, larynx, tonsil, salivary gland and esophagus.

Immunohistochemistry was done with pancytokeratin and leukocyte common antigen to confirm the histopathological diagnosis, 2 cases were found to be positive for lymphoma panel and reported as lymphoma.

Pancytokeratin positive cases were selected for immunostaining with latent membrane protein- 1 and it was found that LMP-1 was associated with 79% of the cases with nasopharyngeal origin.These results are consistent with Raab-Traub et al and Chang et al[64, 65].

Of LEC among other sites, salivary gland LEC showed strong association with LMP -1, LEC in pyriform fossa and posterior 1/3 of tongue showed weak and moderate staining pattern respectively.LEC in other than these sites showed negative results which are consistent with Bijan Khademi et al[76] indicating LMP-1 association is more specific for LEC of nasopharyngeal origin.

This present study also shows that higher level of LMP-1 expression was associated with younger age group (75%). Thus confirming the earlier study by Abdelmajid Khabir et al[39]

As per literature LMP 1 potentiates a variety of signaling pathways including the nuclear factor kb, Mitogen activated protein kinase,and phophotidylinositol 3 –Kinase Alt pathways and involver is angiogenesis which is a key step in tumor growth and invasion and metastasis[65, 66, 67].

LMP-1 protein in essential for EBV-mediated immortalization of B lymphocytes and its expression has been shown to inhibit the terminal differentiation of keratinocytes, thus providing a possible explanation for the lack of differentiation of most EBV associated epithelial tumor cells.[68]

In addition to oncogenesis, LMP-1 is suggested to be relevant to the metastatic property of NPC[72]. Studies also report that LMP-1 positive NPCs show a more progressive attitude and an increased tendency towards lymphnode metastasis than LMP-1 negative NPCs ,Studies suggest that

induction of c-met proto-oncogene by LMP-1 in mediated by activation of Ets-1 transcription factor which leads to upregulation of cell motility considered to be an essential factor in the multiple steps of metastasis.[69,74]

In this present study 2 cases presented with cervical node metastasis, were associated with high levels of LMP-1 expression. Also a 13 year old boy with LEC who presented with vertebral region metastasis within one year of the diagnosis of the primary tumor and was also found to be associated with high levels of LMP-1 expression.

Interestingly, according to Padhamanathan and collegues[75] LMP-1 was detected in all preinvasive NPCs that quickly developed into invasive NPCs. Thus LMP-1 mediated enhancement of metastatic potential could be an early event in NPC, although regulation of LMP-1 expression in NPC remains to the elucidated.

To confirm, the association of EBV in a given tumor, the virus must be detected with in the tumor cells. EBV latent membrance protein -1 (LMP-1) in usually positive in only 30-40% cases. It is usually less sensitive and specific than other molecular analysis.

However Abdel Majiid Khabir et al observed in his study that no biopsy is completely devoid of LMP-1 positive cells and he also suggested the use of S12 antibody which is more sensitive in staining tissue section than CSI-4 antibody. In this present study we used CSI-4 antibody for detecting the

presence of EBV in tissue sections. This might be the reason for LMP -1 negative LEC of nasopharynx in this study.

Dietz et al concluded that tyramid augmented IHC for LMP-1 staining was found to be clearly positive in samples previously showed negative staining with conventional IHC. Both the studies showed 100% LMP-1 expression.

TABLE - 10

Reference	Year of study	No.of LMP-1 positive samples	Tumor tissue source
Plaza et al(43)	2002	13/30	Paraffin block
Dietz et al(77)	2004	33/33	Paraffin block
G Bar Sela et al(78)	2004	6/45	Paraffin block
Abdel majiid et al	2005	29/29	Paraffin block
Present study	2009	15/25	Paraffin block

Despite the limitations of LMP-1, its simplicity, applicability to paraffin sections and its use as an indicator of progressiveness of the tumor has made it an attractive ancillary method for early diagnosis of lymphoepithelial carcinoma.Metastatic potential of LEC might be reduced by inhibition of the LMP-1 mediated signaling pathways.

SUMMARY AND CONCLUSION

During the period of study from May 2006 to September 2009 tumors which were diagnosed as undifferentiated carcinoma (34 cases) were taken up for study.

LEC of nasopharynx showed bimodal distribution with peaks in 10-20 years and 60-80years of age group.

There was a male predominance as previous studies

Nasopharynx was the commonest site for LEC.

Cytokeratin has proved to be the most specific and sensitive marker for confirming LEC.

All cytokeratin positive cases again stained with LMP-1.79% of LEC were found to be associated with LMP-1 expression.

LEC of nasopharyngeal origin showed strong association with EBV than LEC of other sites.

Younger age group showed higher levels of LMP-1 expression. Also patients who presented with metastasis, showed higher levels of LMP-1 expression, thus adding weight to the metastatic potential of LMP-1 expression.

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